EXHIBIT B

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Atherogenic High-Fat Diet Reduces Bone Mineralization in Mice

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ABSTRACT

The epidemiological correlation between osteoporosis and cardiovascular disease is independent of age, but the basis for this correlation is unknown. We previously found that atherogenic oxidized lipids inhibit osteoblastic differentiation in vitro and ex vivo, suggesting that an atherogenic diet may contribute to both diseases. In this study, effects of an atherogenic high-fat diet versus control chow diet on bone were tested in two strains of mice with genetically different susceptibility to atherosclerosis and lipid oxidation. After 4 months and 7 months on the diets, mineral content and density were measured in excised femurs and lumbar vertebrae using peripheral quantitative computed tomographic (pQCT) scanning. In addition, expression of osteocalcin in marrow isolated from the mice after 4 months on the diets was examined. After 7 months, femoral mineral content in C57BL/6 atherosclerosis-susceptible mice on the high-fat diet was 43% lower (0.73 ± 0.09 mg vs. 1.28 ± 0.42 mg; $p = 0.008$), and mineral density was 15% lower compared with mice on the chow diet. Smaller deficits were observed after 4 months. Vertebral mineral content also was lower in the fat-fed C57BL/6 mice. These changes in the atherosclerosis-resistant, C3H/HeJ mice were smaller and mostly not significant. Osteocalcin expression was reduced in the marrow of high fat-fed C57BL/6 mice. These findings suggest that an atherogenic diet inhibits bone formation by blocking differentiation of osteoblast progenitor cells. (J Bone Miner Res 2001;16:182–188)

Key words: osteoporosis, oxidized lipids, bone, atherosclerosis, high-fat diet

INTRODUCTION

EPIDEMIOLOGICAL EVIDENCE links osteoporosis with cardiovascular disease, independently of age.[1,2] Osteoporosis and the subsequent 1 million fractures in the United States each year[3] results from a combination of increased bone resorption and decreased bone formation. Low bone mineral density (BMD) is associated closely with cardiovascular disease mortality,[4–6] cardiovascular calcification,[7–9] atherosclerosis,[10,11] and high lipid levels.[10–13] Such correlations raise the possibility of a common underlying factor or mechanism.

We previously found that minimally oxidized low-density lipoprotein (MM-LDL), and other bioactive oxidized lipids that promote atherogenesis and are increased in atherosclerotic lesions,[14–19] also inhibit osteoblastic differentiation of bone- and marrow-derived preosteoblasts in vitro.[20,21] Preosteoblasts harvested from the marrow of mice fed a high-fat, atherogenic diet showed significantly less osteoblastic differentiation.[21] Others have shown a paucity of cells committed to the bone lineage in osteoporotic bone marrow and with aging.[22,23] These links between lipids, vascular disease, and bone suggest the novel hypothesis that oxidized lipids are the biological link.

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In this study, effects of a high-fat atherogenic diet versus control chow diet on bone mineral content (BMC) and BMD were tested in two strains of mice with genetically different susceptibility to oxidized lipids and atherogenesis. In 1985, Paigen et al. showed differences in the susceptibility of two inbred strains of mice to development of hyperlipidemia and atherosclerotic lesions when fed an atherogenic diet[24,25]; C3H/HeJ were identified as a resistant strain and C57BL/6 as a sensitive strain. Several years later, Liao et al. reported the induction of inflammatory genes by an atherogenic diet in the C57BL/6 but not in the C3H/HeJ strain,[26] and Navab et al.[27] and Shih et al.[28] found differences in the antioxidant defense systems between the susceptible and resistant mouse strains. In the present study, we have compared the susceptibility and resistance of these two strains of mice to the effects of high-fat diet-induced hyperlipidemia on bone. We report that in the atherosclerosis-susceptible C57BL/6 mice, BMC and BMD were significantly lowered by the high-fat diet versus chow diet. These changes were smaller in the atherosclerosis-resistant C3H/HeJ mice. In addition, marrow cells from the high-fat–fed C57BL/6 mice showed reduced osteocalcin expression.

Altogether these results suggest that oxidized lipids adversely affect bone by inhibiting osteoblastic differentiation. If applicable to humans, these studies may result in new therapeutic approaches to osteoporosis.

MATERIALS AND METHODS

Mice and diets

At 1 month of age, male C57BL/6 (atherosclerosis-susceptible strain) and C3H/HeJ (atherosclerosis-resistant strain) mice (The Jackson Laboratory, Bar Harbor, ME, USA) were placed on either a control chow diet (National Institutes of Health [NIH]-31 Mouse/Rat Diet 7013 containing 6% fat) or a high-fat (atherogenic) diet (Teklad TD90221; Harlan Teklad, Madison, WI, USA; including 1.25% cholesterol, 15.8% fat, and 0.5% cholate). This atherogenic diet has been found to cause significant hypercholesterolemia in C57BL/6 mice.[24,25] Femurs and lumbar vertebrae were harvested from 8 animals after 4 months and 14 animals after 7 months. The bones were cleared of soft tissue and fixed in 95% ethanol.

Quantitative computed tomographic scanning

Peripheral quantitative computed tomographic (pQCT) scans were performed on individual bones (left femur, L4 vertebrae) from each mouse. Scanning was done with a STRATEC XCT 960M unit (Norland Medical Instruments, Ft. Atkinson, WI, USA) specifically configured for small bone specimens. Mineral thresholds were set at 1.30 for low-density bone and 2.00 for high-density bone. These thresholds excluded mouse fat, water, muscle, and tendon from true bone. Daily calibration was performed with a manufacturer-supplied phantom (hydroxyapatite in Lucite) of defined density. Calibration with a set of known hydroxyapatite standards (0.05–1000.0 mg/mm^3) yielded a correlation of 0.998 with XCT 960M estimation of volumetric density. Estimates of measurement precision of mineral and volume of femurs and vertebrae were obtained from the middiaphyseal shaft of a B6C3H-F1 femur and from the midbody scans of a B6C3H-F1 L5 vertebra. Six replicate measurements for each bone yielded average values of 1.6, 2.1, and 2.8% for femoral density, mineral, and volume, respectively, and 3.2, 5.9, and 4.7% for L5 vertebral density, mineral, and volume, respectively.

Femurs were scanned full length at 2-mm intervals with a resolution of 0.100 mm/voxel, yielding eight 1-mm-thick cross-sections representing eight axial levels of the femur. Vertebrae were scanned full length at 0.7-mm intervals with the same resolution, yielding three to four 1-mm-thick cross-sections. The center-most scan (based on image morphology) or the mean of two scans sharing the center position was selected for data analyses.

Marrow isolation

After 4 months on the diets, mouse marrow cells were isolated from both femurs from 2 animals in each group as previously described.[21,29,30] Marrow from both femurs was pooled for each animal and RNA was isolated and analyzed separately by reverse-transcriptase polymerase chain reaction (RT-PCR). RNA was isolated as previously described using the RNA isolation kit from Stratagene (La Jolla, CA, USA).[28]

RT-PCR

RNA in 3-μg quantities was reverse-transcribed, and PCR was performed using primers as described previously.[31] Thermal cycling was carried out for 21 cycles (glyceraldehyde-3-phosphate dehydrogenase [GAPDH]) or 34 cycles (osteocalcin) at 60°C annealing temperature for both GAPDH and osteocalcin. Amplified fragments were isolated on a 6% polyacrylamide gel (29:1 acrylamide to bis-acrylamide), and the autoradiographs were scanned with an AGFA ARCUS II scanner and semiquantitated with NIH Image software, version 1.59, public domain program (National Institutes of Health, Bethesda, MD, USA).

Lipoprotein preparation and oxidation

Human LDL was isolated by density-gradient centrifugation of serum and stored in phosphate-buffered 0.15 M NaCl containing 0.01% EDTA. MM-LDL was prepared by iron oxidation of human LDL as previously described.[20] Minimal oxidation of LDL resulted in a 2- to 3-fold increase in conjugated dienes and 2–3 nmol of thiobarbituric acid reactive substances per milligram of cholesterol after dialysis. The concentrations of lipoproteins used in this study are reported in micrograms of protein. The pre- and postoxidation lipopolysaccharide levels in these lipoprotein preparations were <30 pg/ml.

Statistical analysis

Differences in BMC and BMD were assessed using Student's two-tailed *t*-test, allowing for unequal variances and unequal sample sizes where appropriate.

TABLE 1. QCT BONE PARAMETERS FOR FEMURS FROM C57BL/6 MICE AFTER 7 MONTHS ON A CONTROL CHOW OR HIGH-FAT DIET

	Mineral content (mg)		*Chow versus high fat*	*Mineral density (mg/mm³)*		*Chow versus high fat*
Slice	*Chow*	*High fat*	p	*Chow*	*High fat*	p
1	2.29 ± 0.82	0.74 ± 0.19	0.002	0.502 ± 0.05	0.441 ± 0.04	0.01
2	1.05 ± 0.21	0.53 ± 0.20	0.001	0.365 ± 0.05	0.355 ± 0.05	0.70
3	1.02 ± 0.08	0.77 ± 0.10	0.0001	0.447 ± 0.05	0.400 ± 0.06	0.02
4	0.99 ± 0.07	0.72 ± 0.06	0.0003	0.440 ± 0.02	0.391 ± 0.02	0.05
5	1.19 ± 0.14	0.78 ± 0.07	<0.0001	0.520 ± 0.04	0.410 ± 0.03	0.01
6	1.26 ± 0.12	0.86 ± 0.10	<0.0001	0.573 ± 0.04	0.465 ± 0.05	<0.0001
7	1.32 ± 0.16	0.75 ± 0.19	0.0008	0.515 ± 0.05	0.416 ± 0.04	0.1
8	1.13 ± 0.43	0.71 ± 0.31	0.001	0.538 ± 0.03	0.476 ± 0.05	0.01
Mean ± SD	1.28 ± 0.42	0.73 ± 0.09	0.008	0.488 ± 0.07	0.419 ± 0.04	0.03

Scans were performed at 8 longitudinal axis positions (slices) for each femur with 1 being most proximal and 8 most distal. Values of BMC and BMD are expressed as mean ± SD over all animals in each diet group.

TABLE 2. QCT BONE PARAMETERS FOR FEMURS FROM C3H/HeJ MICE AFTER 7 MONTHS ON A CONTROL CHOW OR HIGH-FAT DIET

	Mineral content (mg)		*Chow versus high fat*	*Mineral density (mg/mm³)*		*Chow versus high fat*
Slice	*Chow*	*High fat*	p	*Chow*	*High fat*	p
1	2.73 ± 0.79	2.00 ± 0.84	0.12	0.596 ± 0.06	0.542 ± 0.06	0.11
2	1.60 ± 0.24	1.26 ± 0.15	0.01	0.510 ± 0.06	0.426 ± 0.06	0.016
3	1.72 ± 0.16	1.40 ± 0.13	0.002	0.800 ± 0.03	0.720 ± 0.05	0.005
4	1.88 ± 0.24	1.66 ± 0.16	0.07	0.883 ± 0.07	0.909 ± 0.03	0.39
5	2.01 ± 0.18	1.73 ± 0.16	0.009	0.853 ± 0.03	0.846 ± 0.02	0.63
6	2.28 ± 0.27	2.06 ± 0.14	0.09	0.922 ± 0.06	0.911 ± 0.02	0.68
7	2.01 ± 0.41	1.95 ± 0.15	0.75	0.694 ± 0.11	0.807 ± 0.09	0.05
8	1.47 ± 0.40	1.55 ± 0.21	0.67	0.612 ± 0.07	0.562 ± 0.04	0.15
Mean ± SD	1.96 ± 0.40	1.70 ± 0.29	0.59	0.734 ± 0.15	0.715 ± 0.19	0.26

Scans were performed at 8 longitudinal axis positions (slices) for each femur with 1 being most proximal and 8 most distal. Values of BMC and BMD are expressed as mean ± SD over all animals in each diet group.

RESULTS

Femoral BMC and BMD

After 4 months, femoral BMD was significantly lower in fat-fed C57BL/6 mice at three of the eight levels scanned ($p < 0.04$; from 0.488 ± 0.038 mg/mm³ to 0.423 ± 0.043 mg/mm³). All three levels were in the middiaphyseal region where variance caused by anatomic complexity is minimized. BMC was not significantly different between the two groups.

After 7 months, femoral BMC was significantly lower in fat-fed C57BL/6 mice compared with control chow-fed mice at all eight levels scanned. Mean mineral content was lowered 43% (from 1.28 ± 0.42 mg to 0.73 ± 0.09 mg; $p \leq 0.002$; Table 1) on the high-fat diet. Changes in mineral content were most significant ($p \leq 0.0003$) at the four middiaphyseal levels (scans 3–6). Femoral mineral density was also significantly lower in fat-fed C57BL/6 mice compared with chow-fed mice at six of eight levels, with a 14.5% mean difference (from 0.488 ± 0.066 mg/mm³ to 0.419 ± 0.035 mg/mm³; $p = 0.03$; Table 1).

In C3H/HeJ mice, which are resistant to the atherogenic effects of a high-fat diet and lipid oxidation products,[24,25] the high-fat diet had less effect on bone mineralization. After 4 months on the diet, C3H/HeJ mice showed no significant difference in femoral BMC at any of the eight levels examined (data not shown); BMD was significantly lower at one of eight scanned sites ($p = 0.01$).

After 7 months on the diet, the fat-fed C3H/HeJ mice had significantly ($p \leq 0.01$) lower BMC compared with chow-fed mice at only three of eight levels (Table 2). However, the overall mean difference for all eight levels did not reach statistical significance ($p = 0.59$). There also was no significant effect of the high-fat diet on femoral mineral density ($p = 0.26$; Table 2).

Lumbar vertebral mineral content and mineral density

At 4 months, there was no significant difference between chow and high-fat diet groups in either vertebral mineral content or density in either mouse strains. However, at 7

TABLE 3. QCT BONE PARAMETERS FOR L4 VERTEBRAE FROM C57BL/6 AND C3H/HeJ MICE AFTER 7 MONTHS ON A CONTROL CHOW OR HIGH-FAT DIET

Total bone				*Cortical bone*			
Mineral content (mg)		*Mineral density (mg/mm³)*		*Mineral content (mg)*		*Mineral density (mg/mm³)*	
Chow	*High fat*	*Chow*	*High fat*	*Chow*	*High fat*	*Chow*	*High fat*
			C57BL/6				
1.20 ± 0.10	0.77 ± 0.10	0.229 ± 0.02	0.212 ± 0.03	0.317 ± 0.08	0.088 ± 0.05	0.455 ± 0.01	0.445 ± 0.01
p	<0.001		0.30		<0.001		0.09
			C3H/HeJ				
1.41 ± 0.35	1.31 ± 0.14	0.248 ± 0.03	0.217 ± 0.01	0.624 ± 0.25	0.445 ± 0.13	0.481 ± 0.02	0.474 ± 0.01
p	0.49		0.03		0.12		0.48

Values are for the central slice or slices for each of the L4 vertebrae and are expressed as mean ± SD over all animals in each diet group.

months, vertebral mineral content was significantly lower in the C57BL/6 fat-fed mice (Table 3). Total mineral content of the central section or sections was lower by a mean of 35% (from 1.2 ± 0.1 mg to 0.77 ± 0.1 mg; $p < 0.001$), primarily because of changes in high-density cortical bone (i.e., a 72% decrease). Total mineral density decreased 7% on the high-fat diet, but this change was not statistically significant. In C3H/HeJ mice, a 7% decrease in total mineral content was found, as well as a 29% decrease in cortical mineral content. These changes did not reach statistical significance. Total mineral density of vertebrae from C3H/HeJ mice decreased 12.5% on the high-fat diet (from 0.248 ± 0.03 to 0.217 ± 0.01 mg/mm³; $p = 0.03$).

Gene expression in marrow cells

After 4 months on the high-fat or chow diets, the marrow isolated from 2 C57BL/6 mice on each diet was analyzed for the expression of three markers of osteoblastic differentiation: alkaline phosphatase, bone sialoprotein, and osteocalcin. All three markers were expressed by the marrow cells. Of the three, only osteocalcin expression was affected by diet, showing a 35% reduction with the high-fat diet when normalized to GAPDH values (Fig. 1).

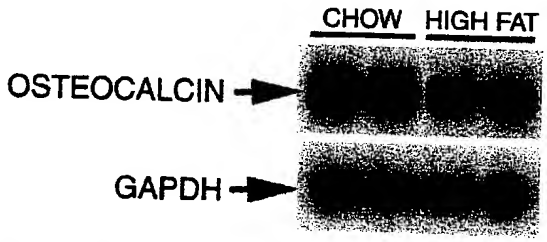

FIG. 1. Effects of a high-fat diet on osteocalcin expression in marrow cells. One-month-old C57BL/6 mice were placed on a high-fat or chow diet for 4 months. The animals were killed and femoral marrow was isolated from each mouse and used to isolate total RNA. RT-PCR analysis showed an expected size band of 360 base pairs (bp). Expression of GAPDH was used for normalization. Each lane represents RNA isolated from an individual mouse.

DISCUSSION

The present study is the first to show that 7-month treatment with an atherogenic high-fat diet lowers BMD and BMC in vivo in atherosclerosis-susceptible C57BL/6 mice, with much smaller effects in the atherosclerosis-resistant C3H/HeJ mice. The atherogenic diet resulted in a significantly lower femoral mineral content and femoral mineral density in the C57BL/6 mice. Smaller changes were seen in the C3H/HeJ mice. The differential effects of the atherogenic diet on bones in the two strains of mice are similar to the effects of that diet on the development of atherosclerosis. Previous reports showed differences in genetically determined factors in response to diet-induced hyperlipidemia and lipid oxidation in these mouse strains to be the underlying reason for their degree of susceptibility to atherosclerosis. These differences include: (1) the level of induction of inflammatory genes such as monocyte chemotactic protein-1, colony-stimulating factors, heme oxygenase, and serum amyloid A and activation of nuclear factor κB (NFκB) transcription factor in response to atherogenic diet[26,27,32] and (2) the ability of high-density lipoprotein (HDL) to protect against the effects of atherogenic diet, because of variability in the level of antioxidant enzyme paraoxonase.[28] The latter difference is important in light of the observation that the protective effect of HDL appears to correlate inversely with atherosclerosis,[33] and a direct correlation between HDL levels and BMD in fat-fed mice has been shown (T. Drake, University of California, Los Angeles [UCLA], Department of Pathology, personal communication, 1999). It is intriguing to speculate that similar genetically regulated factors, involved with defense against atherogenic oxidized lipids, also determine susceptibility to osteoporosis.

Because femoral mineral content was more substantially changed by the atherogenic diet than mineral density, the effect may be caused by quantitatively less bone formation

and/or shorter bones in the high-fat–fed mice. Although we did not measure femoral size after 7 months in this study, in a separate study, we found no significant change in the femoral or tibial length between chow-fed versus high-fat–fed C57BL/6 mice after 4 months on the diet (F. Parhami, unpublished observations, 1999). Because our previous in vitro and in vivo studies showed inhibition of osteoblastic differentiation and bone formation by marrow stromal cells isolated from C57BL/6 mice on the high-fat diet versus chow diet, we speculate that bone formation is inhibited by the atherogenic diet. More direct future studies will further validate this speculation. It is important to note that the mice used in the present study were in their growing stage when peak bone mass is achieved. Inhibition of bone formation during growth stage also would have adverse consequences by reducing peak bone mass. The reducing effects of the dietary fat on BMC and BMD would translate into a reduction in this important determinant of bone strength.

The present results also suggest that increased dietary lipids interfere with osteoblast maturation in vivo, based on dietary inhibition of osteocalcin messenger RNA (mRNA) expression. Although the effect of the high-fat diet on the expression of osteocalcin alone is not sufficient to draw definitive conclusions about differentiation of osteoblasts, this inhibition is consistent with previous ex vivo evidence that exposure to a high-fat diet reduced marrow preosteoblastic maturation in culture,[21] as well as in vitro evidence that lipid and lipoprotein oxidation products inhibit osteoblast differentiation and function.[20,21] Previous studies using the same atherogenic diet in C57BL/6 mice have shown 2- to 3-fold increases in cholesterol levels after 3–4 weeks on this diet, as well as a significant drop in the HDL levels.[24,25] We therefore speculate that the adverse effects of the high-fat diet on bone in the C57BL/6 mice are caused by dyslipidemia and subsequent increases in lipid oxidation. The diet-induced hyperlipidemia in circulation further translates into increased lipid accumulation in highly vascular tissues and the artery wall because of the diffusion of lipoproteins across the vascular endothelium. Once apart from the protective, antioxidant environment of serum, these lipoprotein particles are oxidized further into biologically active forms responsible for inflammatory processes in atherosclerosis and vascular calcification.[19,20] Because bone and marrow are both vascularized, circulating lipids can access both sites of active bone remodeling where osteoprogenitor cells are present: (1) the subendothelial space of the osteons and (2) the marrow stroma at the trabecular surface or endosteum. Lipid accumulation[34] and monocyte accumulation and plaquing[35] have been observed in the vessels of osteons in osteoporotic and aging bone. The presence of circulating lipoproteins in the marrow is expected because marrow is a site for clearance of chylomicrons and chylomicron remnants derived from dietary fat,[36] and dietary fat has been found to alter the lipid profile in the marrow.[37] Thus, lipid oxidation products may underlie the paradoxical association of cardiovascular disease with osteoporosis.

The findings in the present report are consistent with a preliminary report showing a significant correlation between dietary cholesterol intake and vertebral bone loss in women,[38] as well as with population studies showing an association of cholesterol levels with osteoporosis in women[13] and, preliminarily, in men.[39] Recent evidence suggests that 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins), lipid-lowering agents commonly used to treat cardiovascular disease, have potent positive effects on bone formation in rodents,[40] and statin therapy in humans correlates with reduced osteoporosis.[41–43] Although the mechanism is proposed to be a direct stimulation of osteoblasts, an equally likely mechanism is an indirect effect through lipid-lowering, given that the dominant site of action of these agents, in both humans and rodents,[46] is in the liver where statins are mostly cleared from circulation.

Evidence suggests that the atherogenic nature of the high-fat diet is essential for effects on bone. Wohl et al. previously showed a minimal effect on BMC of a noncholesterol, 8% fat diet in adult roosters.[47] Because cholesterol feeding is necessary to induce atherosclerosis in roosters,[48] this finding suggests that a nonatherogenic high-fat diet is not sufficient to induce bone changes.

Collectively, these observations suggest the adverse effects of lipids on bone. The possibility that lipid oxidation products are the biologically active factors linking a high-fat diet with reduced bone formation is supported by the finding of substantially reduced effects in mice that are resistant to the effects of oxidized lipids and by the anabolic effects of the antioxidant vitamin E on bone.[49] Because cardiovascular disease is the highest risk cause of death for patients with osteoporotic fracture[4,5] and low BMD is associated with mortality independent of fractures,[50] elucidation of common lipid- and lipid oxidation–mediated mechanisms has great importance for identifying new preventive measures for both osteoporosis and cardiovascular disease. The possibility that high lipid levels are a common underlying factor in atherosclerosis and bone loss may explain the epidemiological evidence for correlation between cardiovascular disease and osteoporosis.

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